Time sensing by NAADP receptors

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NAADP (nicotinic acid–adenine dinucleotide phosphate) is a newly described intracellular messenger molecule that mediates Ca^{2+} increases in a variety of cells. However, little is known of the mechanism whereby ligand binding regulates the target protein. We report in the present paper that NAADP receptors from sea urchin eggs undergo an unusual stabilization process that appears to be dependent upon the time during which receptors are exposed to their ligand. We demonstrate that receptors ‘tagged’ with NAADP for short periods were more readily dissociated following subsequent delipidation than those labelled for longer. Stabilization of NAADP receptors by their ligand was delayed relative to ligand association taking on the order of minutes to develop at picomolar concentrations. The stabilizing effects of NAADP did not require cytosolic factors or the continued presence of NAADP and persisted upon solubilization. NAADP receptors, however, failed to stabilize at reduced temperature. We conclude that NAADP receptors possess a simple molecular memory endowing them with the remarkable ability to detect the duration of their activation.

Key words: calcium, desensitization, memory, nicotinic acid–adenine dinucleotide phosphate (NAADP), sea urchin egg, time sensing.

INTRODUCTION

In many cells, changes in cytosolic Ca^{2+} concentration are governed by the actions of intracellular Ca^{2+}-mobilizing messengers produced in response to extracellular stimulation [1]. In addition to InsP_{3} [1] and cADP-ribose [2], NAADP (nicotinic acid–adenine dinucleotide phosphate) has recently emerged as an important regulator of Ca^{2+} dynamics [3]. NAADP-sensitive Ca^{2+} channels may be expressed on novel acidic Ca^{2+} stores [4–7] that are distinct from the endoplasmic reticulum which houses receptors for InsP_{3} and ryanodine/cADP-ribose [1]. Additionally, NAADP receptors may be located in or closely associated with the plasma membrane to mediate Ca^{2+} influx [8]. Several studies have shown that NAADP can initiate complex Ca^{2+} signals by interacting with other intracellular Ca^{2+}-mobilizing messengers [7,9–12]. Such ‘chatter’ [13] may be indirect by NAADP providing a ‘trigger’ release of Ca^{2+} which is subsequently propagated via InsP_{3} and/or or ryanodine receptors either by Ca^{2+}-induced Ca^{2+} release [9] or through the overloading of endoplasmic reticulum Ca^{2+} stores [14]. Alternatively, NAADP may have more direct effects on ryanodine receptors [15–18]. Regardless of its exact mechanism of action, what is clear is that NAADP can modulate important Ca^{2+}-dependent events, including neurotransmission [19–21] and neurite extension [7].

NAADP receptors have been reported to undergo a novel form of desensitization whereby channels can inactivate before activation (reviewed in [22]). In sea urchin eggs, where the effects of NAADP were first characterized [23,24], low concentrations of NAADP (<1 nM) which are below the threshold for detectable Ca^{2+} release are able to inhibit subsequent challenge with a normally maximal concentration of NAADP [25,26]. These effects are relatively slow to develop, graded with respect to NAADP concentration, and last indefinitely in broken preparations but slowly reverse in intact cells [27]. In several mammalian cells too, the concentration-effect relationships for NAADP-mediated Ca^{2+} release are bizarre [9]. In T-lymphocytes, for example, the response to NAADP is concentration-dependent in the nanomolar range, but is progressively attenuated at higher concentrations, such that little Ca^{2+} release is detectable in the micromolar range [28]. Such ‘bell-shaped’ responses have also been reported in pancreatic β-cells [29] and a related cell line [30]. Although the mechanistic basis of these effects and the physiological significance are not known at present, one consequence of this unusual behaviour is a marked increase in the dynamic range of messenger action.

Binding of NAADP to its receptor in sea urchin egg homogenates is unusual in that it is not readily reversible [25,31,32]. This property might underlie the novel kinetics of inactivation in this preparation described above, although in low-K^{+} medium where binding is reversible, NAADP receptors are still inactivated by subthreshold concentrations of NAADP [33]. Tight interaction of NAADP with its target protein under appropriate experimental conditions has been exploited to define some of the basic biochemical properties of the NAADP-binding protein [34]. NAADP receptors have been ‘tagged’ with radiolabelled NAADP in their native membrane environment and solubilized while still liganded, thus allowing convenient tracking of the target protein during biochemical manipulation [34]. Results of gel filtration and native gel electrophoresis have indicated that NAADP is associated with a molecular complex ~450 kDa in size with an pI of ~6 [34]. These complexes, however, appear somewhat buoyant following density-gradient centrifugation, due possibly to their association with phospholipids which are required for ligand binding [35]. The target protein has yet to be isolated, and consequently our understanding of how ligand binding regulates the receptor is limited.

In the present study, we provide evidence that binding of NAADP to its target protein in sea urchin eggs is followed by a temperature-sensitive process that renders receptor complexes less prone to dissociation. Our data indicate that NAADP receptors have the intrinsic ability to determine how long they had been exposed to their ligand.

Abbreviations used: IM, intracellular-like medium; NAADP, nicotinic acid–adenine dinucleotide phosphate.

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associated radioactivity was determined. Cold 15
4m g / m l
%

precipitating the extracts at room temperature (20–22°C) to allow the base-exchange reaction. [32P]NAADP was purified by separation of the final mixture by anion-exchange chromatography exactly as described in [31], and fractions were adjusted to pH 7.2 by the addition of 200 mM Hepes.

Labelling of NAADP receptors

Sea urchin (Lytechinus pictus) egg homogenates (2.5%, w/v) prepared as described in [33] were incubated for 5–120 min either at room temperature or at 4°C in an IM (intracellular-like medium) composed of 250 mM potassium gluconate, 250 mM N-methyl-d-glucamine, 1 mM MgCl2, and 20 mM sodium/Hepes (pH 7.2, with acetic acid) supplemented with the indicated concentration of [32P]NAADP (50–1000 pM). In some experiments, egg homogenates were pre-treated with 100 nM staurosporine (Sigma) or a phosphatase inhibitor cocktail (Calbiochem; 1:100 dilution as directed) for 15 min before the addition of calcium. In other experiments, egg homogenates were first washed three times by centrifugation at 100000 g for 5 min at 4°C. After labelling, unbound radiolabel was removed by centrifuging three times at 100000 g for 5 min at 4°C, and the homogenates were resuspended at 32% (v/v). Aliquots of 5 µl were removed, and the extent of labelling of the membranes was quantified by Cerenkov counting. Labelled homogenates were diluted 4-fold and solubilized with 1% (w/v) Triton X-100 for 60 min at 4°C. Insoluble material was pelleted by centrifugation at 100000 g for 60 min at 4°C, and the supernatant containing tagged soluble NAADP receptors was recovered and stored at 4°C before use.

Soluble NAADP receptors that had been labelled previously with [32P]NAADP were recovered by centrifugation following precipitation of protein was independent of subthreshold concentration of NAADP for a further 10 min, and release of 44Ca+ was initiated by the addition of 10 µM NAADP for 2 min. The 44Ca+ content of the intracellular stores was determined by rapid filtration of the homogenate under vacuum. Filters (Whatman GF-B) were washed three times with ice-cold IM supplemented with 3 mM LaCl3. Non-specific binding of 45Ca2+ to sea urchin egg homogenates was determined by the addition of the Ca2+ ionophore ionomycin (10 µM; Calbiochem).

RESULTS

Time-dependent effects of NAADP on NAADP receptor elution

Soluble NAADP receptors that had been labelled previously with their ligand were analysed by gel filtration (Figure 1A). As shown in Figure 1B, much of the ligand was co-eluted with apoferritin (440 kDa), consistent with our previous size determinations of NAADP receptor–ligand complexes [34]. Strikingly, when the time of labelling of receptors with ligand was decreased from 60 min (Figure 1B) to 5 min (Figure 1C), substantially less ligand was recovered in its bound form. This clear time-dependence was quite remarkable, given that NAADP receptors were exposed to their ligand several hours before fractionation (Figure 1A).

We have demonstrated previously a requirement for phosphoryl lipid in order for NAADP receptors to bind their ligand such that, under delipidating separation conditions (identical with those employed in the present study), NAADP receptor–ligand complexes partially dissociate [35]. We considered the possibility that the difference in the observed elution pattern as a consequence of labelling time was due to differential susceptibility of the receptor to delipidation. In support, when separation was performed in the presence of exogenous phospholipids, ligand dissociation was prevented and no difference was observed in the recovery of ligand between preparations labelled for 5 and 60 min (Figure 1D). These results show clearly that receptor–ligand complexes labelled for different times are intact up to the point of gel filtration. Indeed, the recovery of ligand from the solubilized preparation following precipitation of protein was independent of the incubation period (Figure 1E). Taken together, these results indicate that only when NAADP receptors are delipidated is the time of labelling revealed. NAADP receptors can therefore ‘remember’ how long they had been exposed to their ligand.

The effects of NAADP on NAADP receptor elution are slow to develop and are concentration-dependent

We quantified the recovery of liganded receptors following different incubation periods (Figure 2). As shown in Figure 2A, longer incubations led to progressively more ligand remaining associated with its receptor. Intriguingly, the extent of labelling, i.e.
Slow stabilization of NAADP receptors by their ligand

Figure 1  Time-dependent effects of NAADP on NAADP receptor elution

(A) Schematic protocol outlining the preparation and analysis of NAADP receptor–ligand complexes. Sea urchin egg homogenates were incubated with [32P]NAADP for various times in an IM, unbound radioactivity was removed by centrifugation, and labelled membranes were incubated with detergent. Soluble receptor–ligand complexes were recovered by centrifugation, and samples were fractionated by gel filtration. Time frames for the various steps are indicated in minutes. (B–C) Elution profile of tagged receptors that had been labelled with radioligand for 60 min (B) or 5 min (C). The peak fraction in which apoferritin elutes is indicated by \( \bigtriangledown \). (D) Separation of samples labelled for the indicated time in gel-filtration medium supplemented with 1 mg/ml phosphatidylcholine (PC). (E) Quantification of the proportion of NAADP that was bound before fractionation. In these experiments, radioactivity associated with pellet fractions was determined following precipitation of the labelled soluble samples. Results are means ± S.E.M. for three experiments.

The relatively slow effects of NAADP described in the present paper are consistent with receptor stabilization involving an enzymatic step(s). Indeed, the effects of NAADP on receptor stability were markedly temperature-sensitive (Figure 3). When labelling was performed at 4°C instead of at room temperature, NAADP receptors failed to stabilize (Figures 3A and 3B). Thus, following a 2 h incubation with 100 pM NAADP at 4°C, only 16 ± 4% (n = 3) of the ligand remained bound to its receptor following gel filtration. This value is similar to that following a 5 min incubation with the ligand (12 ± 3%; n = 3). Importantly, total ligand bound to the homogenates, was not different following the different incubation times (Figure 2A). Thus steady-state binding was achieved relatively rapidly. Clearly then, the rate at which NAADP receptors become ‘stabilized’ is substantially lower than the rate at which receptors bind their ligand. The absolute rate of stabilization, however, was highly dependent on the concentration of ligand during labelling. At 1 nM [32P]NAADP, receptors appeared to be maximally stabilized by 5 min; recovery of high-molecular-mass complexes was no different following short or long incubations. In contrast, at 50 pM [32P]NAADP, the half time for stabilization was 24 min. Steady-state binding was proportional to NAADP concentration and was achieved by 5 min in all experiments (Figure 2C).
the extent of labelling at 4°C and room temperature was not different following short or longer incubations (Figure 3C), indicating that reducing the temperature affected only the stabilization of the receptor by its ligand and not ligand binding itself. Paradoxically then, reducing temperature results in decreased receptor stability. Stabilization of NAADP receptors by their ligand, however, was unaffected by pre-incubation of the homogenates with non-specific kinase inhibitor staurosporine or a phosphatase inhibitor cocktail (Figure 3D). Washing of homogenates by centrifugation before labelling also did not affect NAADP-mediated receptor stability, indicating that cytosolic factors (including ATP added during preparation of homogenates) were not required (Figure 3D). When egg homogenates were first solubilized and then exposed to their ligand, soluble NAADP receptors underwent stabilization similarly to membrane-bound receptors (Figure 3E). Again, as with membranes, steady-state binding (determined by precipitating labelled samples before gel filtration) was achieved by 5 min (Figure 3G). However, the proportion of liganded receptor recovered after gel filtration following a 60 min incubation with NAADP was clearly greater than following a 5 min incubation (Figure 3F). Taken together, the above results suggest that the ability to distinguish the time of labelling is probably an intrinsic property of the target protein.

**NAADP receptors stabilize in the absence of free ligand**

The lack of correlation between the kinetics of stabilization and ligand binding led us to consider that the former may be mediated by a second reversible, possibly low-affinity, binding site. Such a site would not be detected by our methods, since any bound ligand would dissociate during washing. To address this, homogenates were labelled with ligand (100 pM) for 5 min, washed and then warmed to room temperature for 55 min before solubilization and gel filtration (Figure 4A). Under these conditions, recovery of bound high-molecular-mass complexes (31 ± 4% ; n = 3) was similar to that when receptors had been labelled with ligand for 60 min before washing (37 ± 3%; n = 3) (Figure 4B). As expected, recovery of liganded receptors following a 5 min incubation period was substantially lower (21 ± 2%; n = 3) (Figure 4C). These results show that NAADP receptors can stabilize in the absence of free ligand, ruling out the possibility of reversible interactions underlying the effects of NAADP.

**NAADP-mediated stabilization is likely to be unrelated to NAADP-mediated inactivation of Ca²⁺ release**

Stabilized forms of the receptor may correspond to previously described inactivated receptor conformations that are unable to mediate Ca²⁺ release [25,26]. We therefore examined the effects of NAADP on intracellular Ca²⁺ store content. In control experiments performed at room temperature, NAADP (10 µM) reduced the Ca²⁺ content of egg homogenates by 32 ± 3% (n = 3) relative to the Ca²⁺ ionophore ionomycin (Figure 5A). Although 1 nM NAADP did not affect Ca²⁺ content, the response of the same homogenate to 10 µM NAADP added 10 min later was inhibited by 97 ± 7% (n = 3) (Figure 5A). These experiments confirm that subthreshold concentrations of NAADP can maximally desensitize NAADP receptors [25,26]. Essentially similar results were obtained when NAADP challenges were performed at 4°C following prior loading of Ca²⁺ stores at room temperature (Figure 5B). Analysis of full concentration–effect relationships indicated that the concentration of NAADP during pre-incubation causing half-maximal inhibition of the response to 10 µM was 0.6 ± 0.3 and 0.34 ± 0.03 nM (n = 3) at room temperature and 4°C respectively (Figure 5C). As demonstrated (Figure 3), NAADP receptors fail to stabilize at reduced temperature. Thus the time-dependent effects of NAADP on receptor stability appear not to underlie the ability of NAADP to inactivate its receptor.

**DISCUSSION**

In the present paper, we describe a novel form of molecular memory likely to be intrinsic to the NAADP receptor which manifests as a stable receptor–ligand interaction. Our results
Slow stabilization of NAADP receptors by their ligand

Sea urchin egg homogenates were labelled at 4°C with 100 pM [^32P]NAADP for 120 (A) or 5 (B) min before solubilization and fractionation by gel filtration. (C) Pooled data from three experiments showing the extent of labelling at 4°C (closed bars) and room temperature (RT; open bars). (D) Stabilization of NAADP receptors by their ligand is unaffected by pre-treatment (15 min) with 100 nM staurosporine, a phosphatase inhibitor cocktail (1:100 dilution, as directed) or washing of homogenates before [^32P]NAADP exposure. Results are means ± S.E.M. for three experiments. (E–G) Analysis of NAADP receptors that had been labelled with [^32P]NAADP after the homogenates were solubilized (sol). (E) Gel-filtration profiles following labelling for 5 (●) or 60 (○) min. (F and G) Proportion of radioactivity remaining bound to its receptor after (F) and before (G) gel filtration. The latter was determined at the indicated time by precipitation of soluble extracts with poly(ethylene glycol) and quantification of radioactivity associated with pellets. Results are means ± S.E.M. for three experiments.

indicate that ligand binding initiates a temperature-sensitive process that renders the target protein less susceptible to delipidation. The observed degree of dissociation is a reliable index of the time during which NAADP receptors were previously exposed to their ligand.

Egg preparations labelled for different times behave quite differently upon fractionation. Clearly reducing the period of labelling reduces the recovery of ligand associated with high-molecular-mass complexes. Several lines of evidence indicate that dissociation of ligand occurs during fractionation and not before. For example, recovery of ligand after precipitation of labelled samples before fractionation was independent of labelling time (Figure 1E). Moreover, the distribution of ligand associated with lower-molecular-mass fractions was broad and very unlike free NAADP, which was eluted in two fractions (results not shown). These results are consistent with the gradual dissociation of ligand from the receptor upon movement of the sample through the column. Finally, no dissociation was apparent when fractionation was performed in the presence of phosphatidylcholine (Figure 1D). These results are significant and again indicate that receptor–ligand complexes under the different labelling conditions are intact before fractionation. More importantly, they suggest that it is only under delipidating conditions that the duration of receptor activation is revealed. It remains to be established whether the time of labelling affects regulation of NAADP binding by other means.

Following exposure of homogenates to their ligand, the ligand was removed by centrifugation, the homogenates were treated
with detergent and the sample was clarified before fractionation. Processing which was performed at reduced temperature took ∼150 min, yet the homogenates labelled for short periods were clearly distinguishable from those labelled for longer. These observations very much point to the NAADP receptor possessing a simple form of molecular memory. It is difficult to establish whether we are underestimating the effects of NAADP on stability, since processing time could not be reduced. However, storing samples for up to 4 h after processing did not affect the distribution patterns upon separation (results not shown).

Although reducing the incubation temperature had little effect on binding of NAADP to its target protein, consistent with our previous analysis [31], it effectively abolished the ability of the receptor to stabilize. Thus most of the ligand was dissociated from the preparation during fractionation even following prolonged incubation at 4°C. These results suggest the involvement of some form of enzymatic process in mediating the effects of NAADP. However, protein kinases and phosphatases are unlikely to be involved, since broad-specificity kinase and phosphatase inhibitors had little effect on the ability of NAADP to stabilize its target. Cytosolic factors also appear not to be involved, since washing of homogenates before the addition of ligand did not affect stabilization. Notably, receptors that had been exposed to their ligand after they had been solubilized retained their ability to sense the time of labelling. It is therefore tempting to speculate

Figure 4  NAADP receptors stabilize in the absence of free ligand

Gel-filtration analysis of egg homogenates that had been labelled with [32P]NAADP for 5 (A and C) or 60 (B) min. After removal of unbound ligand, the homogenates were incubated at room temperature (RT) for an additional 55 min (A) or maintained at 4°C (B and C) before solubilization and fractionation. Results are means ± S.E.M. for three experiments.

Figure 5  Effects of temperature on inactivation of Ca2+ release by sub-threshold concentrations of NAADP

Egg homogenates were loaded to steady state with 45Ca2+, and the 45Ca2+ content of the intracellular Ca2+ stores was determined following no additions (−) or addition of 10 µM ionomycin (Iono), the indicated concentration of NAADP or a maximal NAADP concentration following pre-treatment with a subthreshold concentration of NAADP (1 nM → 10 µM). Homogenates were challenged at either room temperature (RT) (A) or 4°C (B). (C) Full concentration-effect relationships for inhibition of NAADP-induced Ca2+ release by non-releasing concentrations of NAADP at the two temperatures. Results are means ± S.E.M. for three experiments.
that if temperature-sensitivity is due to enzyme involvement, then this activity may be intrinsic to the NAADP-binding protein.

Perhaps the most striking aspect of our results is the mismatch between the rate of association of ligand binding and the rate of receptor stabilization. Stabilization clearly occurs after steady-state binding had been achieved. This apparent discrepancy is unlikely to be due to occupancy of a reversible binding site, since receptors are able to stabilize in the complete absence of free ligand (Figure 4). Rather, it appears that NAADP receptors bind their ligand relatively rapidly and that this process is followed by some slow change that renders them less likely to dissociate (Figure 6). The latter, however, is not an ‘all or none’ phenomenon, because the rate of receptor stabilization was clearly concentration-dependent. Of note is the fact that, although binding of NAADP receptors is irreversible, the extent of labelling was graded (Figure 2). This is most likely to be due to the receptor concentration exceeding ligand concentration under the present experimental conditions [31]. The marked dependence of receptor stabilization on ligand concentration might therefore reflect an interaction between independent liganded receptors.

The slow time-dependent effects of NAADP on receptor stability reported here are similar in some respects to the slow inactivating effects of non-releasing concentrations of NAADP on Ca\(^{2+}\) release [25,26]. As we have shown, stabilization is prevented at 4 °C, whereas inactivation is not (Figure 5). The latter results are consistent with those from a previous study demonstrating that NAADP was able to inactivate NAADP receptors at 7 °C [38]. We also demonstrate that the rate of stabilization was very much concentration-dependent. In contrast, the rate (but not extent) of inactivation of Ca\(^{2+}\) release by NAADP appears to be largely insensitive to ligand concentration [25,26]. Stabilization and inactivation are therefore unlikely to be directly related. It therefore remains to be established what functional role stabilization plays. What is clear is that at low concentrations of ligand, where stabilization takes tens of minutes to develop fully, the receptor is likely to be inactivated. It is notable that, although inactivation of Ca\(^{2+}\) release is essentially permanent in egg homogenates, the inhibitory effects do reverse slowly in intact cells [27]. One possibility then is that stabilization might reflect some change in receptor conformation occurring during prolonged receptor occupancy involved in receptor resensitization, although clearly in the homogenate, this is abortive. Alternatively, stabilization might modulate Ca\(^{2+}\) release at higher activating concentrations of NAADP. Clearly, this feature is not essential for channel opening, since Ca\(^{2+}\) release is observed at reduced temperature. However, it is possible that, since it precedes ligand binding, it may reflect desensitized conformations of the target protein. Since the receptor can be experimentally trapped in this conformation (by lowering of the incubation temperature), parallel kinetic analysis of Ca\(^{2+}\) release and detergent susceptibility would be most informative and could provide novel insight into the molecular events that occur upon ligand binding.

Several studies have provided evidence for direct activation of mammalian ryanodine receptors by NAADP [15–18]. In the present study, we have identified a novel feature of the NAADP target protein in sea urchin eggs. To the best of our knowledge, we are unaware of any similar modulation of ryanodine (or InsP\(_3\)) receptors by any described ligand. We therefore argue that the NAADP receptor in our system is a unique protein, which remains to be identified at the molecular level. Indeed, we confirm our previous findings that NAADP receptors are substantially smaller than defined intracellular Ca\(^{2+}\) channels [33–35], although we cannot rule out that the immediate target for NAADP is a smaller accessory protein that interacts with the Ca\(^{2+}\) channel that is responsible for Ca\(^{2+}\) release. We reiterate that, in the sea urchin egg homogenate, the Ca\(^{2+}\)-mobilizing effects of NAADP are unaffected by prior desensitization of the ryanodine receptor by ryanodine/ADP-ribose and by several ryanodine receptor antagonists [24,39]. Although it is conceivable that NAADP and ADP-ribose act on the ryanodine receptor at distinct sites, such a notion is not supported by the physical segregation of Ca\(^{2+}\) stores that are responsive to the two agonists upon density-gradient centrifugation [23]. The effects of NAADP in the sea urchin egg homogenate are thus most simply explained by the existence of a novel intracellular Ca\(^{2+}\)-release channel.

In summary, we provide evidence that NAADP receptors can effectively remember how long they had been exposed to their ligand. This novel property highlights further the complex nature of this most unusual Ca\(^{2+}\)-release channel.

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**REFERENCES**


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14 Churchill, G. C. and Galiane, A. (2001) NAADP induces Ca\textsuperscript{2+} oscillations via a two-pool mechanism by priming IP\textsubscript{3} - and cADPr-sensitive Ca\textsuperscript{2+} stores. EMBO J. 20, 1–6


37 Chini, E. N. and Dousa, T. P. (1996) Nicotinate–adenine dinucleotide phosphate-induced Ca\textsuperscript{2+} release does not behave as a Ca\textsuperscript{2+}–induced Ca\textsuperscript{2+}–release system. Biochem. J. 316, 709–711
